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(54) Title: IN-SITU HYBRIDIZATION IN SUSPENSION FOR DETECTION OR SEPARATION OF CELLS

(57) Abstract

A process is provided for detecting nucleic acid sequences within a cell in suspension. A technique for directly hybridizing labelled deoxyoligonucleotides with cellular DNA or RNA sequences contained in suspended, intact cells is disclosed along with techniques for separating cells containing hybridized DNA or RNA from cells that do not contain hybridized DNA or RNA.

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IN-SITU HYBRIDIZATION IN SUSPENSION FOR DETECTION OR SEPARATION OF CELLS

BACKGROUND OF THE INVENTION

This invention relates generally to nucleic acids and more specifically to techniques for hybridizing and detecting nucleic acid sequences within a cell while the cell is in suspension.

Complementary strands of nucleic acid such as DNA or RNA can form a double-stranded molecule linked by complementary bases in a process known as hybridization. Hybrids may be formed between any two complementary strands, so hybrids of DNA:DNA are possible, as are DNA:RNA and RNA:RNA. The reaction is reversible under particular temperature and salt concentration conditions. Most often, nucleic acids are first isolated and then permitted to hybridize with particular labelled nucleotide sequences termed probes. While such nucleic acid hybridization is well known in the art, methods for hybridization of nucleic acid sequences within cells in suspension have not been available.

In situ hybridization of nucleic acid in cells affixed to slides and coverslips has been described by Gall, J. and Pardue, M., Proc. Nat. Acad. Sci., (USA), 63:378-83 (1969). These early attempts at in situ hybridization used, as probes, naturally occurring DNA or RNA purified from cells or cell supernatants and labelled with radioisotopes. Because of limited sensitivity, however, only highly reiterated, or amplified, genes were susceptible to the technique.

Nucleic acid hybridization techniques have since been simplified by using recombinant DNA or synthetic oligonucleotides as probes. See, for example, Venesky, D. et al., Cell, 24:385-391 (1981) and Montgomery, E. A., et al, Cell, 14:673-80 (1978). Labelling probes with nonradioactive compounds has further streamlined the methodology, Rudkin, G.T. and Stollar, B.D., Nature, 265:472-73, (1977), Langer, P. R. et al., Proc. Natl. Acad. Sci. (USA) 78:6633-37 (1981); Jablonski, et al., Nucleic Acids Research 14:6115-28 (1986).

In addition, in situ hybridization has been used to detect DNA sequences within suspended nuclei removed from cells, Trask, B., et al., Science, 230:1401-03, (1985), Trask, B., et al., Human Genetics, 78:251-59 (1988), Van der Engh, G.J. and Trask, B.J. (1988), U.S. Patent No. 4,770,992. Trask, et al. cross-linked nuclei, isolated from cells, with dimethylsuberimidate to prevent disintegration of the nuclei during denaturation and hybridization. N-acetoxy-2-acetylaminofluorene (AAF), its iodinated analog AAIF, and biotin-labelled chromosomal and cloned DNA probes were hybridized to their respective targets and the probes detected with anti-AAF or anti-AAIF antibodies and a secondary rhodamine-labelled antibody (for AAF- or AAIF-labelled probes) or fluorescein-avidin-DSC (for biotin-labelled probes) by flow cytometry.

These methods of hybridizing isolated nucleic acid, or nucleic acid in immobilized cells or suspended nuclei, have various applications, for example in the detection of the presence of infectious agents. Nevertheless, such methods have serious limitations. For example, they do not allow the detection of nucleic acid in the cytoplasm

of the cells. Often only a small percentage of the cells in a sample contain the nucleotide sequence of interest. Detecting the presence of even a small number of infected cells can be of critical importance, however. Where the nucleic acid from a sample is isolated and pooled, the dilution by non-complementary sequences from uninfected cells can preclude detection of infected cells. On the other hand, where hybridization is performed on immobilized cells, detecting positive cells by microscopy can be burdensome, time-consuming, and unreliable.

Flow cytometry is a technique for rapidly sorting individual cells, microorganisms and cell organelles into groups based on observable differences. Briefly summarized, the technique involves passing a stream of material to be measured (cells, lysed cells or other material) in single file past a light source where the particular property is detected, usually based on the reaction of the light to the materials. A computer then directs the separation of the material as desired. This technique is very rapid and allows separation of from 10^4 to 10^6 cells per minute.

Fluorochromes are most often used to label the cellular component of interest before passing the cell sample past the light source. A variety of fluorochromes have been used to nonspecifically stain DNA, RNA and proteins for cytometric analysis. Immunological techniques have been used to detect cell surface markers and cell interior markers specifically with fluorescent antibodies.

Nonfluorescent dyes can also be utilized in this system. Cells can absorb light as they pass through a

focused light beam, and light transmitted by cells stained with nonfluorescent dyes can be quantitated and related to analyte concentration. This technique can help measure the expression of various target molecules in cells. A summary of flow cytometry as a technique is provided in Muirhead, K.A., et al., Biotechnology, 3:337-56 (1985).

It has been known for some time that DNA or RNA can be hybridized in cells which have been affixed to slides or another solid support. Further, it has been known that in situ hybridization may be used to detect DNA sequences within suspended nuclei removed from cells. Until now, however, methods for nucleic acid hybridization within cells in suspension had not been available. It was commonly believed that without the structural support of a slide or another solid matrix, cellular components would disintegrate during the rigorous hybridization procedures. See, for example, U.S. Patent No. 4,770,992. Moreover, even if the cells remain intact during the procedures, there is difficulty in both getting the probe inside the cell and nuclear membranes and washing unbound probe out of cells after hybridization.

There thus exists a long-felt need for methods to hybridize probes to nucleic acid in suspended cells. Such a method would be of particular use in conjunction with automated methods for identifying and separating cells, such as flow cytometry. The present invention satisfies this need and provides related advantages as well.

SUMMARY OF THE INVENTION

The present invention provides a technique for the hybridization of nucleotide probes to nucleic acids within a cell while the cell remains in suspension. The technique comprises fixing the suspended cell, hybridizing the nucleic acid within the cell with an oligonucleotide probe and detecting the hybridized probe. The nucleic acid can be denaturated prior to hybridization and endogenous enzymes can be ablated. The invention also provides a technique for categorizing cells by flow cytometry based on differences in target nucleic acid sequence. Additional objects and advantages of the invention will be apparent to those skilled in the art upon reading the specification and claims.

A cell suspended in fluid having a hybridized nucleotide probe therein is also provided as well as a kit for detecting a specific nucleotide sequence in a cell suspended in a fluid. The kit comprises a means for fixing the suspended cell and a nucleotide probe complementary to a nucleotide sequence within the suspended cell. The kit can also include a means for detecting the hybridized probe.

DETAILED DESCRIPTION OF THE INVENTION

A method is provided for detecting a specific nucleotide sequence in the nucleic acid in a cell suspended in a fluid. The method comprises (1) fixing the suspended cell in such a way as to permit a nucleotide probe to penetrate without disrupting the suspended cell, (2) providing a nucleotide probe complementary to the specific nucleotide sequence within

the suspended cell under conditions permitting hybridization, and (3) detecting hybridized probe within the cell, whereby hybridized probe indicates the presence of the specific nucleotide sequence. The method can include the further step of denaturing the nucleic acid before hybridizing with a nucleotide probe or ablating endogenous enzymes.

As used herein, the term probe or nucleotide probe refers to a labelled nucleotide sequence. The nucleotides can be deoxyribonucleotides or ribonucleotides. The number of nucleotides present in the probe is variable, depending on the application. Preferably, the range is between 10 and 2000 nucleotides, more preferably between 15 and 50, most preferably 20 to 25. As used herein, oligonucleotide refers to nucleotide sequences less than 200 residues in length. Various labels are well known in the art and include fluorophores, enzymes, and radioactive isotopes, haptens and other ligands. Methods for making such labelled probes are described, for example, in PCT Publication No. WO 84/03285. Where a probe is used to detect the presence of complementary sequences in a cell in suspension, the probe can be a single selected homogeneous sequence or can be a mixture of various selected sequences. A mixture can be used, for example, to increase the sensitivity of the assay or to permit detection even where a portion of the nucleic acid in the target cell is variant, as a result, for example, of genetic alteration.

The present invention is useful for detecting a target nucleic acid sequence in a single cell in suspension. The technique may be achieved using

automatic flow analysis, for example, flow cytometry. In one embodiment, the technique involves manufacturing oligonucleotides by methods known in the art. See, for example, United States Patent No. 4,500,707 Matteucci and Caruthers, (1981), J. Am. Chem. Soc., 103:3185-3191, which is incorporated herein by reference. These oligonucleotides are then labelled with enzymes, fluorophores or ligands (molecules with high affinity for other molecules), also by means known in the art. See, for example Jablonski, E.G., et al. (1986), Nucleic Acids Research 14:6115-6128; Heller, M.J., et al. (1986), Fed. Proc. 45:1516; PCT Publication No. WO 84/03285, all of which are hereby incorporated by reference. These labelled oligonucleotides may be used as probes for hybridization with DNA or RNA in the nucleus or in the cytoplasm of cells. The presence of hybridized cellular DNA or RNA is then established by detecting the label on the probe.

The in situ hybridization assay set forth here may be used in tandem with flow cytometry to assist for example in the diagnosis of various agents that infect or invade cells (viruses, chlamydia, certain bacteria and certain parasites) and to diagnose cancer and genetic diseases.

Detection by microscopic analysis can be used in place of flow cytometry, but flow cytometry is preferred. The technique of the present invention may be combined with flow cytometry to form an automated, rapid detection or quantitation system for use in hospitals and research laboratories. The technique of the present invention may also be used separately and the labelled cells may be detected in a subsequent step, if desired.

A technique of the present invention may be briefly summarized as follows: (1) oligonucleotide probes are synthesized and labelled with enzymes, fluorophores, ligands or other detectable labels; (2) the probes are then hybridized with DNA or RNA in a fixed suspended cell, using the methods set out below; (3) the labels in the cells are detected using known optical techniques; and (4) the cells are separated, if desired.

In accordance with the invention, synthetic probes, preferably DNA, are prepared using conventional techniques. The probes are designed to be complementary to particular nucleotide sequences, termed target sequences of nucleic acids within the fixed cell. The selection of nucleotide sequence of the probe is within the skill and choice of those in the art. Although long chain probes (greater than 200 nucleotides) can be used and may be more sensitive than shorter probes, synthetic oligonucleotide probes (10 to 200 nucleotides, preferably 15 to 50 nucleotides) are preferred. Since higher concentrations of oligonucleotide probes can be used in the technique of the invention, maximum hybridization can usually occur within a short time, for example, ten minutes or less. Moreover, oligonucleotides may be labelled directly, while long probes must typically be labelled indirectly, adding complexity and expense to the process but without providing significant additional reliability. A directly labelled probe is one where the label to be detected is directly attached to the probe. An indirectly labelled probe is one which has attached to it a moiety through which the label to be detected is subsequently attached. Additionally, short probes can more easily permeate cells than can longer probes.

Examples of labels include enzymes, fluorophores and ligands. Other labels are available and are well known in the art. Enzyme labels include alkaline phosphatase, horseradish peroxidase, ß-galactosidase, glucose oxidase and luciferase. Fluorophores include fluorescein and Texas Red. Ligand labels include biotin, digoxigenin, AAF and AAIF.

Protected linker arm nucleoside 3'-phosphoramidite was prepared by the method of Ruth, J. DNA, 3:123 (1984). The linker arm monomer was incorporated directly into automated oligonucleotide synthesis employing an Applied Biosystems Model 380A DNA synthesizer using the phosphoramidite chemistry on controlled pore glass as first described by Matteucci and Caruthers, Tetrahedron Letters 21:719-722 (1980), which is incorporated herein by reference. The purified oligonucleotide was covalently cross-linked to alkaline phosphatase using the homobifunctional reagent disuccinimidyl suberate through the reactive primary amine on the linker arm by the method of Jablonski et al., Nucleic Acids Research, 24:6115-6128 (1986).

The preferred conditions for nucleic acid denaturation is a combination of heat 50°C to 100°C, preferably 65°-70°C, and a formamide concentration of 40% to 100%, preferably about 70% ultrapure: 30% low salt buffer).

The hybridization technique is useful for <u>in situ</u> hybridization within cells in suspension. Before hybridization can occur, the cell must be fixed. Fixation is the chemical preservation of cells or tissue so that the structure will be minimally altered from the

normal state. There are generally two types of fixatives, crosslinking (for example, paraformaldehyde, glutaraldehyde) and precipitating (alcohols). For suspension in situ hybridization, fixation with paraformaldehyde is preferred, although other crosslinking fixatives will achieve effective results. The preferred amount of paraformaldehyde is between 0.5% and 5%. Precipitating fixatives are less desirable, as cell morphology is not well maintained and cells tend to clump.

After cells are fixed, it may be necessary to ablate endogenous enzyme by treating cells with 0.2N HCl. Treating cells with acid destroys cellular enzymes which may act to turn over alkaline phosphatase substrates, in addition to further permeabilizing cells. In the course of fixation, the cell must be opened enough so that the probe can penetrate the cell, but not enough so that the nucleus and cytosol (including mRNA and viral RNA or DNA) will escape. In addition, unreacted probe can be washed out of the cell to remove background labels yet the cell must still be kept intact to analyze without removing the hybridized sequences.

After cells are fixed, nucleic acid within the cells can be denatured. Denaturing agents or conditions for nucleic acid include formamide, glyoxal, heat (90°C to 100°C, preferably 0 to 0.3M salt concentration), acid or base (DNA only) treatments. Denaturation with a combination of heat (>65°C) and formamide (both DNA and RNA denaturation) is preferred. Where the secondary structure of the nucleic acid does not provide a barrier to hybridization, for example where the nucleic acid is shorter, single stranded RNA or where the probe is short

and recognizes an exposed sequence, denaturation is not necessarily required.

After denaturation, if used, probe in hybridization buffer is added immediately and hybridization commences. Conditions for hybridization depend on probe base content. Hybridization can occur in the presence or absence of formamide (<40%) and in a temperature range of ambient temperature (23°C) to 75°C for alkaline phosphatase probes; the temperature and formamide concentration are experimentally determined. Washing of unreacted or loosely bound probe from cells occurs at a temperature and salt concentration which are likewise experimentally determined (23-75°C,<0.3M). Detection of alkaline phosphatase labelled probe hybridized to its complement in cells occurs preferably with the addition of NBT and BCIP substrates, although other substrates (especially substrates which give insoluble products) can also be utilized. A red to purple product is desired. In situ hybridization of cells, accomplished in suspension, is well adapted to automation and thus is highly desirable for applications where a complete automated system is preferable, for example, for clinical diagnostic purposes.

To illustrate the technique of in situ hybridization of cells in suspension, the following examples are provided. These examples are intended to illustrate the invention rather than to limit its applicability.

EXAMPLE I

Cytomegalovirus (CMV)-infected and uninfected human fetal foreskin fibroblast (HFFF) cells were treated with

0.5% paraformaldehyde in phosphate-buffered saline (PBS), a common buffer, for one minute at 60°C. Treating for ten minutes at ambient temperature (23°C) was an acceptable alternative.

The fixed cells were suspended for two minutes at room temperature with 0.2N HCl. The nucleic acids within the suspended cells were denatured with a mixture of 70% formamide (ultrapure) and 30% 2X SSC (0.3M sodium chloride, 0.03M sodium citrate, pH 7.0) for 10 minutes at 70°C.

The cells were then immediately hybridized by placing them in "hybridization buffer" with a mixture of four 2.5 nM alkaline phosphatase-labelled probes (20-22 bases) specific for CMV for ten minutes at 55°C. "Hybridization buffer" contains 5X SSC (0.75 sodium chloride, 0.075M sodium citrate, pH 7.0), combined with 0.5% bovine serum albumin (BSA, Fraction V). Alternatively, the cells and probe were mixed with 30% formamide in 70% hybridization buffer at 37°C for ten minutes.

CMV sequences are available from GenBank, (IntelliGenetics, Mountain View, CA) Accession Nos. K01090, M10063, M11911). The following nucleotide sequences were used as probes:

1) 5' GGCGAAAAGAAGACGCGTGT 3'
2) 5' TTCTATGGAGGTCAAAACAGCG 3'
3) 5' TGGCCAAAGTGTAGGCTACAAT 3'
4) 5' GGAAAGTCCGAATCCTACACAT 3'

Excess probe was washed out of the cells three times, for 2 minutes each, with 1X SSC (0.15M sodium chloride, 0.015M sodium citrate, pH 7.0) and cells separated from wash by low speed centrifugation at 55°C. Alternatively, cells were washed three times with 0.5X SSC (0.075M sodium chloride, 0.0075M sodium citrate, pH 7.0) at 37°C.

Substrate was added to cells so that the labelling enzyme was detected. A 1 ml solution of 0.33 mg/ml nitro blue tetrazolium (NBT) and 0.17 mg/ml 5-bromo-4-chloro-3-indolylphosphate (BCIP) in alkaline phosphatase (AP) buffer (0.1M Tris-HCl, pH 8.5; 0.1M NaCl, 0.05M $MgCl_2$; and 0.1mM $ZnCl_2$) was pre-warmed to 37°C and added to the cells. Incubation took place for 1.0 hour, during which time the enzyme label converted the substrate to an insoluble purple dye. The cells were rinsed with distilled water and were ready for analysis and/or separation by acceptable methods, including cell cytometry. Cells positive for virus turned an opaque blue-purple, while uninfected cells remained colorless.

EXAMPLE II

Human immunodeficiency virus (HIV) persistently infected and uninfected CEM cells (lymphoid cell line) were treated with 1.0% paraformaldehyde in PBS for ten minutes at ambient temperature. The fixed cells were suspended for two minutes at room temperature in 0.2N HCl. The nucleotides in the suspended cells were denatured with a mixture of 70% formamide (ultrapure) and 30% 2X SSC for 10 minutes at 70°C.

The cells were immediately hybridized by placing them in hybridization buffer with a mixture of twenty 2.5 nM alkaline phosphatase-labelled probes (20-24 bases) specific for HIV for ten minutes at 55°C. HIV sequences are available from GenBank, Accession number K03455. Labelling was as described in Example I. Excess probe was washed out of the cells three times, with 1X SSC at 45°C.

Substrate was added to cells so that the labelling enzyme was detected. A 1 ml solution of 0.33 mg/ml nitro blue tetrazolium (NBT) and 0.17 mg/ml 5-bromo-4-chloro-3-indolylphosphate (BCIP) in alkaline phosphatase (AP) buffer was pre-warmed to 37°C and added to the cells. Incubation took place for 0.5 hours, during which time the enzyme label converted the substrate to an insoluble purple dye. The cells were rinsed with distilled water and were ready for analysis and/or separation by acceptable methods, including cell cytometry. Cells positive for virus turned an opaque blue-purple, while uninfected cells remained colorless.

EXAMPLE III

Herpes simplex virus type 1 (HSV)-infected and uninfected HEp-2 cells were treated with 0.5% paraformaldehyde in PBS for one minute at 60°C. The fixed cells were then suspended for two minutes at room temperature in 0.2N HCl. The cells were further treated, in suspension, with 0.01% hydrogen peroxide in methanol for 20 minutes at ambient temperature.

The nucleic acids in the cells were denatured with a mixture of 70% formamide (ultrapure) and 30% 2X SSP

(0.36M NaCl, 20 mM NaH_2PO_4, pH 7.0) 10 minutes at 70°C. The cells were immediately hybridized by placing them in "hybridization buffer II" with 5 nM horseradish peroxidase-labelled 22 base probe specific for HSV1 and HSV2 for ten minutes at 50°C. The HSV sequence is available from GenBank, Accession No. J02224. Purified oligonucleotide was covalently cross-linked to horseradish peroxidase by the method of Jablonski, et al., Nucleic Acids Research, 24:6115-6128 (1986), which is incorporated herein by reference. "Hybridization buffer II" contained 5X SSP (0.9M NaCl; 50 mM NaH_2PO_4, pH 7.0) and 0.5% BSA (Fraction V). Excess probe was washed out of the cells three times, with 1X SSP (0.18M NaCl; 10mM NaH_2PO_4, pH 7.0) at 50°C.

Substrate was then added to the cells so that the labelling enzyme could be detected. A 1 ml solution of 0.5 mg/ml 0-dianisidine dihydrochloride in HRP buffer (0.1M imidazole, 0.1M NaCl, pH 7.4 with 0.001% hydrogen peroxide) was added to the cells. Incubation took place for 1.0 hour at ambient temperature, during which time the enzyme label converted the substrate to an orange dye. The cells were rinsed with distilled water and were ready for analysis and/or separation by acceptable methods, including cell cytometry. Cells positive for virus turned orange, while uninfected cells remained colorless.

EXAMPLE IV

Herpes simplex virus (HSV)-infected and uninfected HEp-2 cells are treated with 0.5% paraformaldehyde in PBS. The fixed cells are then suspended for two minutes at room temperature with 0.2N HCl.

The suspended cells are denatured with a mixture of 70% formamide (ultrapure) and 30% 2X SC for 10 minutes at 70°C. The cells are immediately hybridized in hybridization buffer with six 5 nM Texas Red-labelled and (separately) six fluorescein-labelled 22-25 base probes specific for HSV1 and HSV2 for ten minutes at 60°C. Purified oligonucleotides were labelled via the linker arm with isothiocyanate or sulfonyl chloride derivatives of either Texas Red or fluorescein by the method of PCT Publication No. WO 84/03285.

Excess probe is washed out of the cells three times, as before, with 1X SSC at 50°C. Cells are ready for analysis and/or separation by acceptable methods, including cell cytometry. Cells positive for virus demonstrate, upon excitation, increased photon emission at the proper wavelength.

It will be readily apparent to those skilled in the art that the methods described in the Examples above may be readily automated. Methods of performing flow cytometry are well known, and various flow cytometers are commercially available. See, for example, Shapiro, H. M., Practical Flow Cytometry, Alan R. Liss, Inc., New York (1988), which is incorporated herein by reference. The hybridized cells may be fed directly into the entrance of flow cytometry instrument, for detection by fluorescence or by optical density, depending on the label used.

Those skilled in the art will recognize that alterations may be made to the invention described above without departing from the scope or spirits thereof.

WE CLAIM:

1. A method of detecting a specific nucleotide sequence in nucleic acid in a cell suspended in a fluid, comprising:

a. fixing the suspended cell in such a way as to permit a nucleic acid probe to penetrate without disrupting the suspended cell;

b. providing a nucleic acid probe complementary to the specific nucleotide sequence within the suspended cell under conditions permitting hybridization; and

c. detecting hybridized probe within the cell, whereby hybridized probe indicates the presence of the specific nucleotide sequence.

2. The method of claim 1, further comprising the step of denaturing the nucleic acid before hybridizing with a nucleic acid probe.

3. The method of claim 1, further comprising the step of inactivating endogenous enzymes.

4. The method of claim 1, wherein the nucleic acid is DNA.

5. The method of claim 1, wherein the nucleic acid is RNA.

6. The method of claim 1, wherein the nucleotide probe is an oligonucleotide probe.

7. The method of claim 6, wherein the oligonucleotide probe is DNA.

8. The method of claim 6, wherein the oligonucleotide probe is RNA.

9. The method of claim 1, wherein the nucleotide probe is labelled with a detectable moiety.

10. The method of claim 9, wherein the detectable moiety is selected from the group consisting of enzymes, fluoropores, or ligands.

11. The method of claim 9, wherein the labelled hybridized nucleotide probe is detected by flow cytometry.

12. The method of claim 9, wherein the labelled hybridized nucleotide probe is detected by microscopy.

13. The method of claim 1, wherein the suspended cells are fixed with a composition comprising paraformaldehyde.

14. The method of claim 1, wherein the suspended cells are fixed with between .01% and 5% paraformaldehyde.

15. The method of claim 1, wherein the suspended cells are denatured with a composition comprising formamide.

16. The method of claim 15, wherein the formamide comprises between 40% and 100% of the composition.

17. The method of claim 1, wherein the fixing is carried out at a temperature between 23° and 75°C.

18. The method of claim 1, wherein the hybridizing is carried out at a temperature between 23° and 75°C.

19. A cell suspended in fluid having a hybridized nucleotide probe therein.

20. A kit for detecting a specific nucleotide sequence in a cell suspended in a fluid comprising a means for fixing the suspended cell and a nucleotide probe complementary to a nucleotide sequence within the suspended cell.

21. The kit of claim 20, further comprising a means for detecting the hybridized probe.

22. A method of detecting a specific nucleotide sequence in nucleic acid in a cell suspended in a fluid, comprising:

a. fixing the suspended cell in such a way as to permit a nucleotide probe to penetrate without disrupting the suspended cell;

b. denaturing the nucleic acid in the cell;

c. providing a nucleotide probe complementary to the specific nucleotide sequence within the suspended cell under conditions permitting hybridization; and

d. detecting hybridized probe within the cell, whereby hybridized probe indicates the presence of the specific nucleotide sequence.

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 90/01191

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC5: C 12 Q 1/68

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
IPC5	C 12 Q

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category *	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
X	WO, A1, 8504720 (HOWARD FLOREY INSTITUTE OF EXPERIMENTAL PHYSIOLOGY AND MEDICINE) 24 October 1985, see the whole document	1-22
X	GB, A, 2139349 (NATIONAL RESEARCH DEVELOPMENT CORPORATION) 7 November 1984, see the whole document	1-22
A	EP, A1, 0127327 (NATIONAL RESEARCH DEVELOPMENT CORPORATION) 5 December 1984, see the whole document	1-22

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

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"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

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IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
20th June 1990	06. 07. 90
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	F.W. HECK

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
A	US, A, 4038143 (ELLIOT JUNI ET AL) 26 July 1977, see the whole document -- --------	20-21

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. PCT/US 90/01191

SA 35529

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 24/05/90 The European Patent office is in no way liable for theseparticulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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